UNIVERSITI PUTRA MALAYSIA

Evaluation of *vacA* and *cagA* Genotypes of *Helicobacter pylori* in Iranian Patients with Peptic Ulcer Disease

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

August 2009

In the Name of God the Compassionate the Merciful

I dedicate this thesis to,
My beloved Father, Mother and Wife for their invaluable love, tolerance, generosity, moral and financial support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

Evaluation of *vacA* and *cagA* Genotypes of *Helicobacter pylori* in Iranian Patients with Peptic Ulcer Disease

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August 2009

Chairman: **Professor Patimah Ismail, PhD**

Faculty: **Medicine and Health Sciences**

Helicobacter pylori infection occurs all over the world, and more than half of the world population is infected by this microorganism. Research on the variety of *H. pylori* genes is valuable from two perspectives; first, for predicting the outcome of the infection and second, for better understanding of its distribution in the world and the evolutionary origins of this organism.

It has been suggested that *Helicobacter pylori* strains containing *cagA* gene and the s1/m1 genotype of vacuolating cytotoxin gene A (*vacA*) may be associated with peptic ulcer diseases. Some studies have also shown that allele s1 of the *vacA* gene is associated with gastroduodenal diseases.

In order to investigate the *cagA* and *vacA* genes, biopsies of the antrum and corpus of the stomachs of patients were obtained. To detect *H. pylori* infection, the *phosphoglucosamine mutase* gene (*glmM*) was amplified through the PCR method and observed on 2% (w/v) agarose gel electrophoresis. All the *H. pylori*-positive samples were subjected to further PCR amplification to determine different alleles of the *vacA* gene. The PCR products were separated on 2% (w/v) agarose gels electrophoresis. 37, 15 and 32 out of 84 specimens were duodenal ulcer (DU), gastric ulcer (GU) and gastritis (GT), respectively. Seventy-seven (91.7%, χ^2= 58.333, $p < 0.05$) out of 84 samples were *H. pylori*-positive. *cagA* gene was detected in 80% (χ^2= 12.6, $p < 0.001$), 76.9% (χ^2= 3.769, $p > 0.05$), and 48.3% (χ^2= 0.034 $p > 0.05$) from DU, GU and GT samples, respectively. It was found that 66% (23/35) of DU samples, 62% (8/13) of GU samples and none of 29 GT samples were s1/m1. 17% (6/35) of DU samples, 15% (2/13) of GU samples and 52% (16/29) of GT samples were s1/m2. 17% (6/35) of DU samples, 23% (3/13) of GU samples and 48% (13/29) of GT samples were s2/m2.

This study demonstrates that the presence of the m2 allele of *vacA* is strongly associated with gastritis and the presence of allele s1 is associated with peptic ulcers. *Helicobacter pylori* strains with *vacA*-s1/m2-*cagA*$^+$ genotype are associated with peptic ulceration diseases.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

Penilaian Genotaip *vac*A dan *cag*A Daripada *Helicobacter pylori* ke Atas Pesakit Iran Yang Dijangkiti Radang Peptik

Oleh

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Infeksi *Helicobacter pylori* berlaku di seluruh dunia dan lebih separuh daripada populasi penduduk dunia dijangkiti dengan mikroorganisma ini. Kajian terhadap berbagai-bagai jenis gen adalah bernilai daripada dua perspektif; pertama, untuk menjangka hasil daripada jangkitan. Kedua, untuk mendapat kefahaman yang lebih mendalam terhadap taburan kepelbagaiannya di dunia dan permulaan evolusi organisma ini.

Adalah dicadangkan bahawa jenis *Helicobacter pylori* mengandungi gen *cagA* dan genotaip s1/m1 sitotoksin genA bervakuol (*vacA*) yang berkemungkinan berkaitan dengan penyakit radang perut. Sesetengah kajian ada menunjukkan alel s1 gen *vacA* adalah berkaitan dengan penyakit radang pangkal usus.

Dalam mengenalpasti gen *cagA* dan *vacA*, biopsi antrum dan korpus perut pesakit diperolehi. Untuk mengesan infeksi, gen *phosphoglucosamine mutase* (*glmM*) telah diamplikasikan menggunakan kaedah PCR dan diperhatikan pada elektroforesis 2% (w/v) gel agar. Semua sampel positif *H. Pylori* ditentukan untuk amplifikasi PCR selanjutnya atau menentukan pelbagai alel gen *vacA*. Hasil daripada PCR telah diasingkan pada elektroforesis 2% (w/v) gel agar. Daripada 84 spesimen, 37 adalah radang duodenum (DU),15 adalah radang gastrik (GU) dan 32 ialah gastritis (GT) . Sebanyak tujuh puluh tujuh (91.7%, χ^2= 58.333, $p < 0.05$) daripada 84 sampels adalah positif *H. Pylori*. Gen *cagA* telah dikesan dalam 80% (χ^2= 12.6, $p < 0.001$) daripada sampel DU, 76.9% (χ^2= 3.769, $p > 0.05$) daripada sampel GU dan 48.3% (χ^2= 0.034 $p >$ 0.05) dari sampel GT. Didapati 66% (23/35) dari sampel DU, 62% (8/13) dari sampel GU adalah s1/m1 dan tiada pada sampel 29 GT dikesan. Sebanyak 17% (6/35) daripada DU sampel, 15% (2/13) adalah sampel GU dan 52% (16/29) sampel GT adalah s1/m2. Sebanyak 17% (6/35) dari DU sampel, 23% (3/13) adalah sampel GU dan 48% (13/29) sampel GT adalah s2/m2.

Kajian ini menunjukkan alel m2 dari *vacA* adalah sangat berkait rapat dengan gastritis dan kehadiran alel s1 adalah berkaitan dengan radang penghadaman. Jenis *Helicobacter pylori* dengan genotaip *vacA*-s1/m2-*cagA*$^+$ adalah berkaitan dengan penyakit radangan penghadaman.

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I certify that an Examination Committee has met on 17-8-2009 to conduct the final examination of Ali Saber Hossein Abadi on his Master of Science thesis entitled "**Evaluation of *vacA* and *cagA* Genotypes of *Helicobacter pylori* in Iranian Patients with Peptic Ulcer Disease**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

_____

ALI SABER HOSSEIN ABADI

Date: 21 September 2009

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LIST OF ABBREVIATIONS

APS	-	Ammonium Persulfate
ASR	-	Age Standardized Ratio
ATP	-	Adenosin Tri-Phosphate
bp	-	Base pair
cagA	-	Cytotoxin Associated GeneA
CAD	-	Coronary Artery Disease
CT	-	Computerized tomography
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic acid
DU	-	Duodenal Ulcer
EDTA	-	Ethylenediamine Tetra-Acetic acid
EtOH	-	Ethanol
GU	-	Gastric Ulcer
GT	-	Gastritis
GERD	-	Gastroesophageal Reflux Disease
H. pylori	-	Helicobacter pylori
kbp	-	kilo base pair
kDa	-	kiloDalton
KAc	-	Potassium Acetate

M	-	Molar
MALT	-	Mucosa–Associated Lymphoid Tissue
mg	-	Milligram
mM	-	Millimolar
mL	-	Milliliter
µg	-	Microgram
µl	-	Microlitre
µM	-	Micrometer
m-region	-	Middle Region
mRNA	-	Messenger RNA
ng	-	Nanogram
OD	-	Absorbance of DNA
OR		Odds Ratio
OMP	-	Outer-Membrane Proteins
PAGE	-	Polyacrylamide Gel Electrophoresis
PAI	-	Cag Pathogenicity Island
PCR	-	Polymerase Chain Reaction
PUD	-	Peptic Ulceration Disease
rDNA	-	Ribosomal DNA
rpm	-	Revolutions per minute
rRNA	-	Ribosomal RNA
s-region	-	Signal Region

SDS	-	Sodium Dodecyl Sulfate
SOD	-	Superoxide Dismutase
SSC	-	NaCl, Trisodium Citrate (Citric Acid)
Taq	-	Thermus aquaticus thermostable DNA
TBE	-	Tris-Borate-EDTA
TE	-	Tris EDTA buffer
TEMED	-	N,N,N',N'-Tetramethyl-Ethylenediamine
tRNA	-	Transfer RNA
V	-	Volt
vacA	-	Vacuolating Cytotoxin GeneA

CHAPTER 1

INTRODUCTION

Peptic ulcer is a gastrointestinal disease which is characterized by mucosal damage secondary to pepsin and excess gastric acid secretion. It usually occurs in the proximal duodenum and stomach. It can also happen in the lower esophagus, the jejunum, and the end of duodenum (Ramakrishnan *et al*., 2007).

Many different factors contribute to gastrointestinal diseases, such as old age, tobacco smoking (Luo *et al*., 2002), the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* infection (Kurata *et al.,* 1997). A collection of other infections such as tuberculosis, Crohn's disease, myeloproliferative disorder, chronic renal failure, and sarcoidosis can be associated with a larger risk of peptic ulce. Critical illness, surgery, or hypovolemia can result in gastroduodenal corrosions or ulcers; these may have some complications such as bleeding or perforation and can be silent (Ziegler 2005).

Active chronic gastritis is the most common type of chronic gastric inflammation that *H. pylori* has been detected in 70% - 80% of cases of this disease (Cover & Blaser 1995). The disease influences the antrum and fundus of the stomach, frequently showing permeation of the lymphocytes and eosinophils. The inflammatory process is more considerable in the antrum than other parts of the body (Atherton *et al*., 1996).

H. pylori can settle in the gastric mucosa and remain there for many years with minimal symptoms and complications in the majority of cases (Blaser *et al*., 1995). It infected about 50% of the world's population (Souto *et al*., 1998). However, it has been reported that some cases have shown significant morphological changes in the gastric mucosa-inflammation to ulceration. Others indicated the progression of chronic gastritis to cancer through chronic atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma (Blaser *et al*., 1995).

The high incidence of *H. pylori* infection may cause around 40 percent of all gastric cancer cases all over the world (Parkin *et al*., 2001). The International Agency for Research on Cancer (IARC) in 1994 reported that there is a relationship between *H. pylori* infection and gastric cancer. They found sufficient evidences that introduced *H. pylori* as a carcinogen among human populations. They classified *H. pylori* as group I definite carcinogen (Chen *et al*., 2007). Yamagata *et al.* (2000) reported a significant relationship between *H. pylori* infection and a succeeding incidence of gastric cancer for men in the Japanese population.

Torres *et al.* showed that about 80% of population is infected by *H. pylori* at an average age of 10 years and 20% of one-year-old children in Mexico had antibodies against *H. pylori* (Torres *et al*., 1998). *H. pylori* Infection has significantly different rates between developing and developed countries. For instance, the annual prevalence of *H. pylori* infection in the United States is 0.5% to 1% in children less than 10 years of age and 50% in adults around 60 years old, respectively. Different ethnic groups, for example Hispanics, Afro-Americans, and Native Americans are

infected at an early period of aging (Everhart *et al*., 2000). Generally, low prevalence of *H. pylori* infection has been observed in the United States, Canada, northern and western Europe, whereas high prevalence of the infection has been reported in India, Africa and Latin America (Torres *et al*., 2000).

The pathogenic effect of *H. pylori* in duodenal ulcer and gastric ulcer is obvious. Eighty percent of patients with gastric ulcer and 95% of patients with duodena ulcer have *H. pylori* infection in the United States (Breuer *et al*., 1998) which is the major cause of peptic ulcer disease in 48% of cases in the U.S. (Ramakrishnan 2007). However, the prevalence of *H. pylori* infection has been reported in about 90% of patients with gastroduodenal diseases in Spain (Arroyo *et al.,* 2004).

A seroepidemiological study in Iran demonstrated that 42.7% of cases between 10 to 25 years old were seropositive for *H. pylori* infection. There was an age-related raise in *H. pylori* infection although there was no significant relationship between *H. pylori* infection and gender (Pirouz *et al*., 2000). Many studies have reported that 85% of the Iranian adult population has *H. pylori* infection (Malekzadeh *et al*., 2004).

Helicobacter pylori is a Gram-negative, spiral, microaerophilic bacterium that chronically infects the gastric mucosa of more than half of the world population. (Parsonnet 1995.) Two putative virulence agents of *H. pylori* have been recognized in ulcerogenic strains. The first one is cytotoxin associated geneA (*cagA*) and the second is vacuolating cytotoxin geneA (*vacA*) (Hsu *et al*., 2002).

A 120-140 kDa protein is encoded by the *cagA* gene and is present in about 60-70% of *H. pylori* strains. This gene is part of the *cag* pathogenicity island (PAI), a 40-kbp fragment with a number of genes contributing to cytokine production (Hsu *et al*., 2002). Strains that do not produce the CagA protein normally lack the whole cag PAI. Current studies have shown that the frequency of *H. pylori* strains with the *cagA* gene is higher in patients with peptic ulcer disease than in patients with chronic gastritis (Guerrero *et al*., 2000).

The vacuolating cytotoxin gene (*vacA*) is another virulence factor in that its product, VacA protein, can damage epithelial cells (Hsu *et al*., 2002). The presence of the *vacA* gene has been proven in almost all *H. pylori* strains and contains two variable parts, the s-region and m-region (Atherton *et al*., 1997). Each of these two regions has the different allelic types, s1 and s2 for the s-region and m-region containing m1 and m2 allelic forms (Van Doorn *et al*., 1998).

Some reports show that type s1 is associated with peptic ulcer disease and it also has a significant relationship with the presence of the *cagA* gene (Rudi *et al*., 1999). m1 allele has been observed more in severe gastric epithelial than m2 allele and it is associated with higher levels of toxin activity (Atherton *et al*.,1997).

There are four combinations of *vacA*; therefore, the genotype s2/m1 was not identified in many studies (Castillo-Rojas *et al*., 2004). Type s1/m1 and s1/m2 strains produce high and moderate levels of toxin, respectively, while strains with the s2/m2 genotype produce little or no toxin (Forsyth *et al*., 1998). Some studies in a number

of countries have demonstrated that *cagA*$^{+}$ and *vacA* type s1 are associated with *H. pylori* causing peptic ulcer disease (Catalano *et al*., 2001). Many publications in Brazil have also proven this relationship (Brito *et al*., 2000). A recent study in Iran revealed that duodenal ulcer has a strong association with *H. pylori* infection (Rasmi *et al*., 2009), whereas another study in Tehran, capital city of Iran, showed no significant relationship between different genotype of *H. pylori* and outcomes (Jafari *et al*., 2008).

Research on the variety of *H. pylori* genes is valuable from two perspectives; first, for predicting the outcome of the infection and second, for better understanding of its distribution in the world and the evolutionary origins of this microorganism. The detection of *vacA* and *cagA*, virulence markers described in several clinical outcomes, can be used to help the treatment and prevention of *H. pylori* infection in Iran. This study investigated different genotypes of *vacA* and *cagA* genes of *Helicobacter pylori* isolated from patients with peptic ulcers and their relationship with this disease.

The objectives of this study were to:

1- Detect *H. pylori* infection by the PCR method in patients with peptic ulcers and gastritis
2- Identify the *vacA* and *cagA* genes in patients
3- Determine the distribution of *cagA* and *vacA* genes among patients with peptic ulcer and gastritis.
4- Investigate the association between *cagA* gene with *vacA* gene and their relationship with gastroduodenal diseases